Technical Note DOER-C8
July 1999

Comparison of Two Cell-Based Assays for Screening Dioxin and Dioxin-Like Compounds in Sediments

PURPOSE: The purpose of this technical note is to report the results of studies comparing two cell-based assays as screening tools for dioxins and related compounds in sediments. The Reuber H4IIE rat hepatoma cell line and the recombinant 101L human hepatoma cell line were used to assay 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) toxic equivalents (TEQs) in split samples of the same sediment extracts. The two methods were compared for sensitivity and correlation with analytical chemistry results. An interlaboratory comparison of results obtained with the 101L cells was performed.

BACKGROUND: The reader is referred to DOER-C1, "Guidance for Performance of the H4IIE Dioxin Screening Assay" (McFarland et al. 1998),[1] for background. In that technical note, the use of cultured mammalian cells as the basis of assays for dioxins in environmental samples was described. The rationale for their use was given, and a detailed laboratory protocol for using the Reuber H4IIE cell line for this purpose was provided. Herein, further studies are reported in which a recombinant cell line, 101L, is used in side-by-side assays with the H4IIE on the same sediment samples.

The basic mechanism that responds to the presence of polychlorinated dioxins/furans (PCDD/PCDF), coplanar polychlorinated biphenyls (PCBs), and polynuclear aromatic hydrocarbons (PAHs) is the same in both H4IIE and 101L cells. The initial reaction is binding of the chemical to a cytosolic receptor protein known as the aryl hydrocarbon receptor (AhR). This association leads to the formation of a stable AhR-ligand complex that translocates to the cell nucleus and binds with dioxin recognition elements (DREs) on the DNA. As a result, certain genes (e.g., CYP1A1) are expressed, and detoxifying enzymes are synthesized. The amount of enzyme produced is directly proportional to the concentration of bound chemical. Hence, the quantitation of one of these enzymes serves as a measure of dioxin activity. In the assay using H4IIE cells, ethoxyresorufin-O-deethylase (EROD) cleaves resorufin as a by-product that can be measured spectrofluorometrically. Recently, advances in transgenic research have produced new ways of detecting dioxins and other chemicals that bind with the AhR. Recombinant cell lines have been developed in which nonmammalian reporter genes are inserted downstream from the DREs in the DNA of the cells. When the DREs are activated, the reporter gene is switched on, producing some protein other than EROD (or another endogenous enzyme) that can be detected instrumentally. For example, the 101L cell line is derived from human hepatoma HepG2 cells and is stably transfected with a plasmid containing the human CYP1A1 promoter sequence fused to the firefly luciferase gene as a reporter (Anderson et al. 1995). The induction of CYP1A1 results in the production of luciferase. Light produced by the action of luciferase on the luciferin substrate can then be measured with a luminometer. The P450 Reporter Gene System (P450RGS) is an assay based on 101L cells that is currently being used

1 *http://www.wes.army.mil/el/dots/doer/technote.html.*

commercially and was the subject for comparisons with the H4IIE cell-based assay (subsequently referred to as EROD assay).

MATERIALS AND METHODS

NYDMMP Sediments. Nine subsamples of sediments collected for the New York Dredged Material Management Plan (NYDMMP) (Simmers and Lee 1998) representing harbors in New York and New Jersey and a composite of the nine samples were tested for dioxin activity using the P450RGS and EROD assays. The sediments were given comprehensive chemical analysis separately. The sediment subsamples were extracted using the Dionex Accelerated Solvent Extraction (ASE™) system under high temperature and pressure following U.S. Environmental Protection Agency (EPA) Method 3540 guidelines for soils and sediments. A portion of each extract was cleaned on a sulfuric acid/silica gel (SA/SG) column to remove PAHs. Both crude and SA/SG-cleaned extracts were solvent-exchanged to iso-octane before testing. Results obtained for both assays were expressed as TCDD TEQs (pg/g) based on 2,3,7,8-TCDD standard curves. Results were plotted against TCDD TEQs (pg/g) generated from gas chromatography/mass spectrometry (GC/MS) chemical analysis data, which were calculated from the summation of TEQs derived from individual congeners of PCDDs/PCDFs and PCBs and/or PAHs.

Miscellaneous Sediments. An additional set of 15 SA/SG-cleaned sediment extracts from samples collected in various other waterways were also analyzed. The samples were extracted by either ASE™ or Soxhlet methods and cleaned on a SA/SG column. The ASE™ and Soxhlet extraction methods have been shown to provide comparable results (McFarland et al. 1998). Results were expressed as described above.

Testing Procedures. The modified testing protocol for the EROD assay in 96-well microtiter plate format was performed according to the protocol described in McFarland et al. (1998). Briefly, 5 µL of a sample extract in iso-octane was added to each well of a 96-well plate containing 5,000 H4IIE cells. Iso-octane controls and a series of TCDD concentrations were also tested. The test mixtures were incubated for 72 hr, after which cells were measured for protein and resorufin simultaneously using a spectrofluorometer.

The detailed procedure for the P450RGS assay is described in American Society for Testing and Materials (ASTM) (1997). Twenty-five thousand 101L cells per well were seeded in six-well plates and incubated for 72 hr. Five microliters of the sample extract in iso-octane were then added to each test well. Iso-octane blanks and TCDD standards were also tested. Following a 6-hr or 16-hr incubation, the cells were washed, lysed, scraped, and the contents transferred into a microcentrifuge tube. After centrifugation, the supernatant was analyzed for luminescence. Fold induction was calculated as the number of times the light units of the test sample exceeded the light units of the iso-octane blanks. The TCDD TEQ of each sample was derived from the TCDD standard curve generated separately. All the results used in making comparisons with the EROD assay were obtained from the 16-hr test. Readings were also taken at 6 hr as a qualitative check on the influence of PAHs relative to the chlorinated compounds in the samples.

RESULTS AND DISCUSSION

Toxic Equivalency. The 2,3,7,8-TCDD congener is the most potent of the 17 PCDD/PCDFs containing the 2,3,7,8-chloro substitution pattern, which is a requirement for AhR binding and for toxicity. For that reason, the concentrations of the other congeners measured in chemical analyses are normalized to 2,3,7,8-TCDD using toxic equivalency factors (TEFs). Toxic equivalents (TEQ) of the mixture are equal to the sum of the concentrations of individual congeners (i) multiplied by their potencies (TEFi) relative to 2,3,7,8,-TCDD.

$$TEQ = \sum(PCDDi \times TEFi) + \sum(PCDFi \times TEFi) \quad (1)$$

Hence, the TEQ of a PCDD/PCDF mixture can be treated as though it were the concentration of 2,3,7,8-TCDD. In addition to these, EPA recognizes 13 coplanar PCBs as having dioxin toxic equivalents.[1]

Coplanar PCBs and some PAHs bind with the AhR similarly to dioxins, but with less strength and less potency to effect gene transcription. TEFs for PCBs and PAHs relative to 2,3,7,8,-TCDD were developed at Columbia Analytical Services, Inc. (CAS), Carlsbad, CA, using the transgenic 101L human hepatoma cells. The CAS TEFs for PCBs and PAHs were included in TEQ calculations for the NYDMMP GC/MS analysis comparisons with TEQs measured in the 101L assay (Equation 2).

$$TEQ = \sum(PCDDi \times TEFi) + \sum(PCDFi \times TEFi) + \sum(PCBi \times TEFi) + \sum(PAHi \times TEFi) \quad (2)$$

Additivity is assumed in the calculation and is a conservative assumption because it is known that some PCB mixtures exhibit nonadditive (antagonistic) interactions (Safe 1994).

NYDMMP Sediments. Results for the SA/SG-cleaned sediment extracts obtained with the P450RGS and EROD assays were plotted individually against the GC/MS chemical analysis data as shown in Figures 1A and 1B, respectively. The TCDD TEQs for the NYDMMP sediment GC/MS data were calculated using PCDD/PCDF and PCB analyses but not PAH analyses, with the assumption that all PAHs were removed by the SA/SG cleanup. Dioxin activity in 2 of the 10 extracts was not detectable with the EROD assay. These two data points were arbitrarily assigned a TEQ of one-tenth the limit of detection (LOD) of the assay. The LOD for the P450RGS assay was 26.8 pg g^{-1}, which was about one-half that of the EROD assay (51.3 pg g^{-1}). The two cell-based assays are compared in Figure 2. Although neither assay correlated with the GC/MS data (r^2 = 0.240 for P450RGS, r^2 = 0.082 for EROD), the correlation between the two assays was good (r^2 = 0.610). Furthermore, the distribution of data points between assays suggests a similar pattern in the two. The P450RGS assay produced a slightly higher response to the cleaned extracts than did the EROD assay.

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1 *http://www.epa.gov/nceawww1/dchem.htm.*

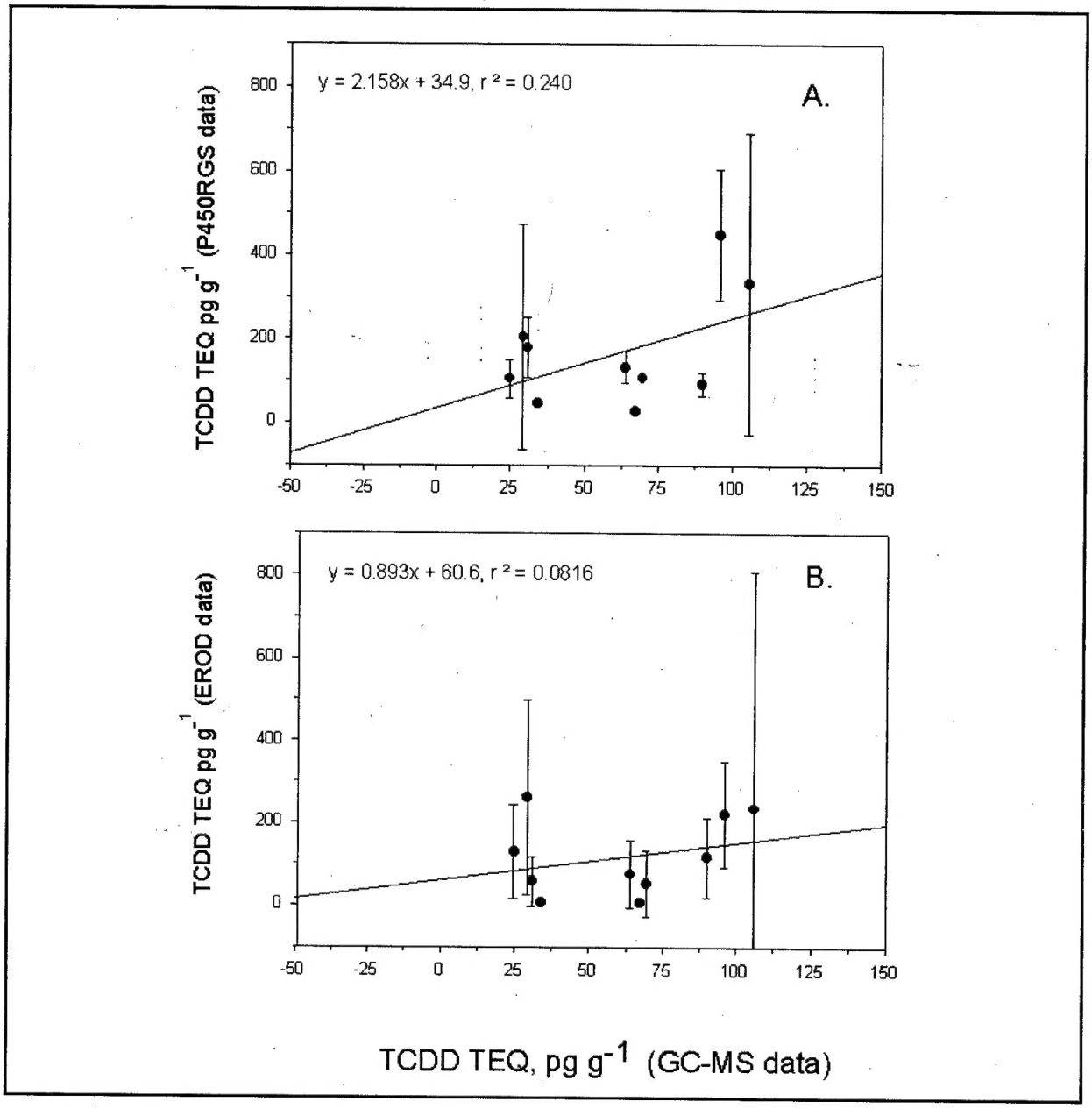

Figure 1. Correlation of calculated TCDD TEQs for GC/MS data with measured TCDD TEQs using the P450RGS assay (A) or the EROD assay (B) on 10 SA/SG-cleaned NYDMMP sediment extracts (Means of six replicates ± SD)

For comparisons of the GC/MS results with cell-based assay results on the crude NYDMMP sediment extracts, the GC/MS TEQs were calculated using PCDD/PCDF, PCB, and PAH data. TEQs of both assays appeared to correlate well with the TEQs calculated from the analytical chemistry data (r^2 = 0.742 and 0.805 for P450RGS and EROD assays, respectively). However, it is obvious that the correlations were strongly influenced by the most contaminated sediment and the clustering of the nine lesser contaminated sediments (Figures 3A, 3B). The same effect is evident in the correlation of TEQs between the two assays (Figure 4). If the influence point is omitted, the correlation is lost for all three comparisons with r^2s ranging only from 0.0012 for the comparison

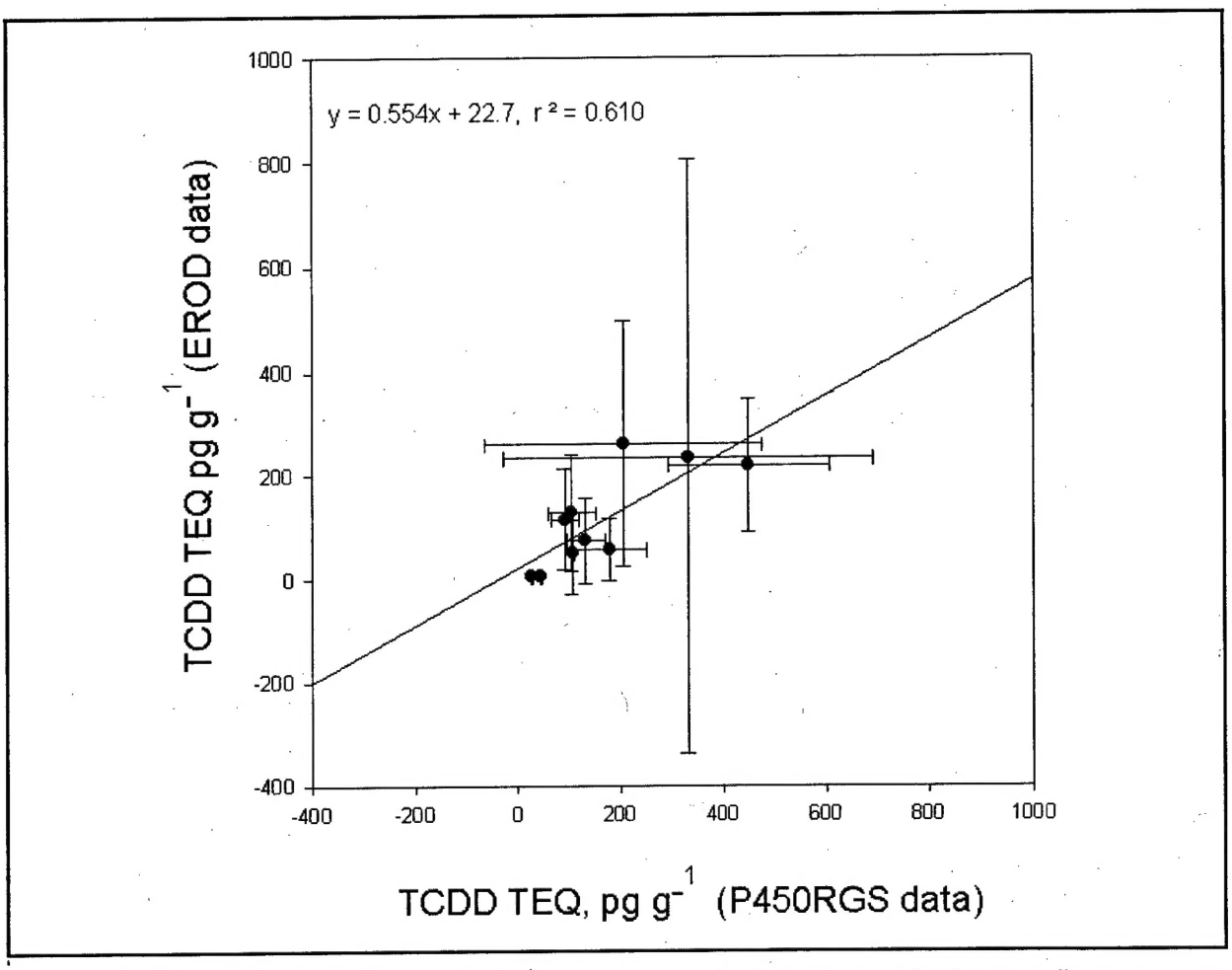

Figure 2. Correlation of P450RGS and EROD assays on 10 SA/SG-cleaned NYDMMP sediment extracts (Means of six replicates ± SD)

of P450RGS TEQs with GC/MS TEQs to 0.148 for the comparison of EROD TEQs with GC/MS. The P450RGS assay produced an approximately 10-fold higher response to the crude extracts than did the EROD assay, indicating a greater sensitivity to PAHs.

The distribution of contributions of the chlorinated and of the PAH contaminants to TEQs in the cell-based assays is shown in Table 1. In the crude NYDMMP sediment extracts, the PCDD/PCDF contributed about one-fourth of the total TEQs with PAHs making up nearly all of the rest. However, in the cleaned extracts, PCDD/PCDF accounted for approximately 97 percent of the total TEQs, assuming that all PAHs were removed with the SA/SG cleanup. Because the PAH content was high, the presence of unknown AhR-active compounds in the crude extracts would not likely affect the calculated GC/MS TEQs significantly. In contrast, the presence of unknown dioxin-like compounds in the cleaned extracts could drastically underestimate the GC/MS TEQs. Such compounds are known to exist and may contribute significantly to toxicity in some environmental media. For example, polybrominated analogs of PCDD/PCDF have similar enzyme induction potencies (Mason et al. 1987), and these have been identified in environmental samples (Sellstrom et al. 1993). The presence of AhR-active compounds in the extracts that were not accounted for in the chemical analysis may partly explain the poor correlations of TEQs by either assay with TEQs from sediment chemistry.

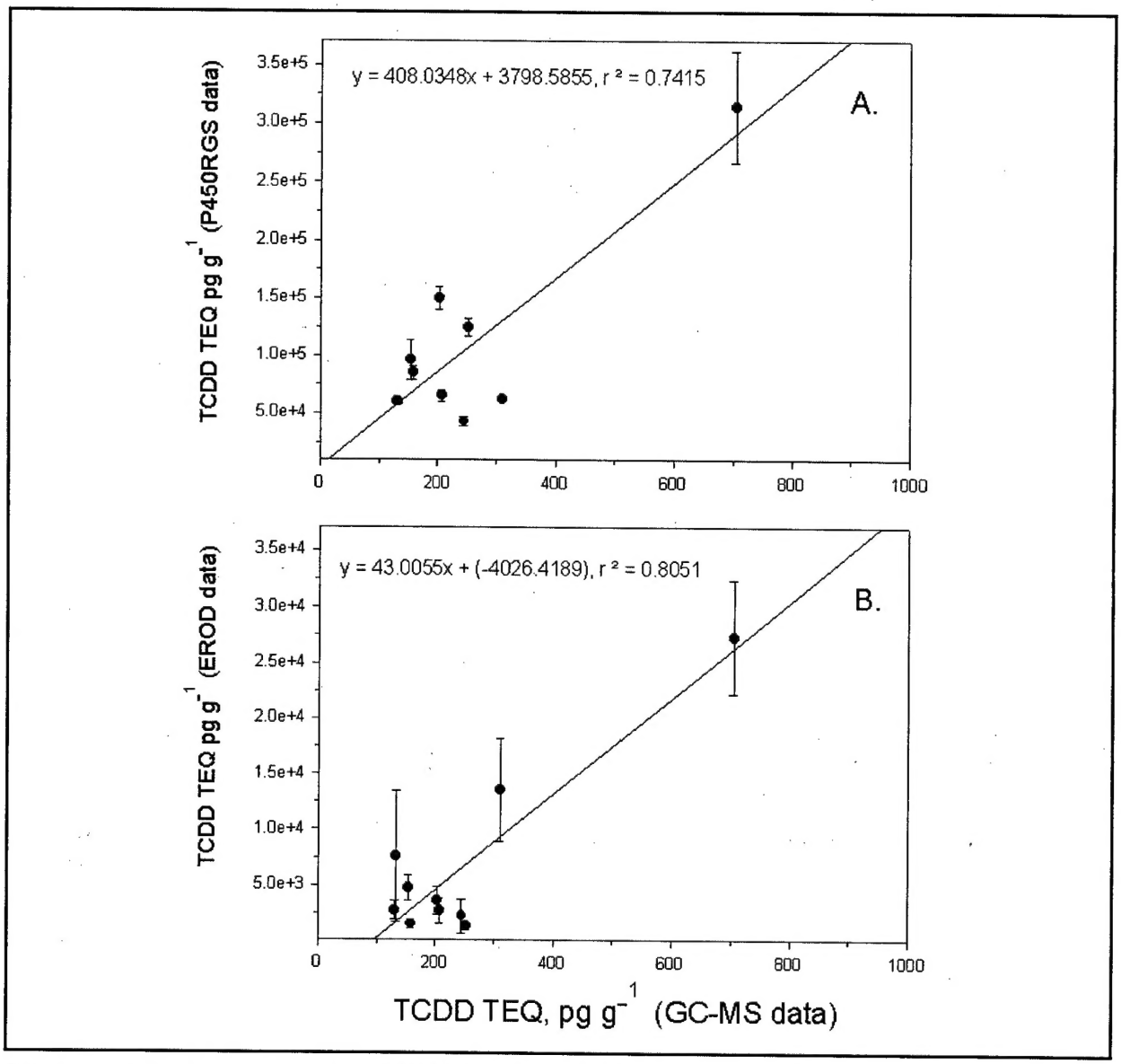

Figure 3. Correlation of calculated TCDD TEQs for GC/MS data with measured TCDD TEQs using the P450RGS assay (A) or the EROD assay (B) on 10 crude NYDMMP sediment extracts (Means of six replicates ± SD)

Miscellaneous Sediments. To further compare the sensitivity of the P450RGS and EROD assays, a set of 15 sediment samples obtained from various other sites were analyzed. Results were expressed and presented as for the NYDMMP sediments. The miscellaneous sediments were not as well characterized by chemical analysis as were the NYDMMP sediments. Only PCDD/PCDF GC/MS chemical analysis was performed on these sediments. One sample, however, was analyzed for both PCDD/PCDF and PCB. In this sample, PCDD/PCDF contributed 99.7 percent of the total TEQs, while PCBs contributed only 0.3 percent (Table 1).

Dioxin activity was detected in all 15 sediment extracts with the P450RGS assay. The correlation between P450RGS and GC/MS data was good ($r^2 = 0.714$) (Figure 5A). Of the 15 samples, only six had detectable dioxin activity in the EROD assay, and the correlation of these six points with

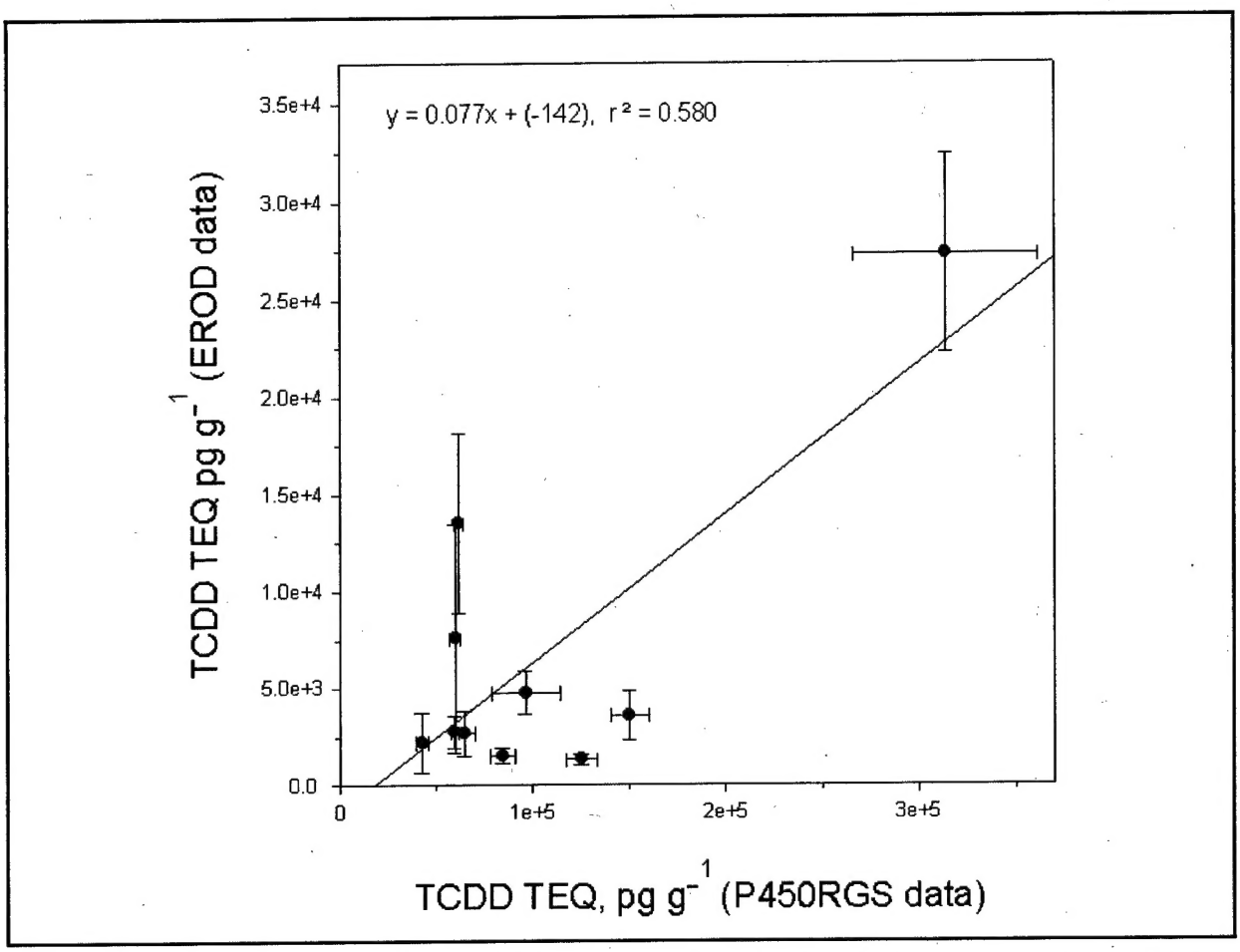

Figure 4. Correlation of P450RGS and EROD assays on 10 crude NYDMMP sediment extracts (Means of six replicates ± SD)

Table 1
Percent Distribution of TEQs in NYDMMP and Miscellaneous Sediment Extracts

Analytes	NYDMMP Sediments		Miscellaneous*
	Cleaned**	Crude	Cleaned**
PCDDs/PCDFs	96.6	23.6	99.7
PCBs	3.4	0.8	0.3
PAHs	NA↑	75.6	NA
Total TCDD TEQs	100.0	100.0	100.0

* TCDD TEQs contributed by PCDDs/PCDFs and PCBs were obtained from one sample. Majority of the miscellaneous samples were not analyzed for PCB content.
**Sulfuric acid/silica gel cleanup step in sample preparation.
↑ Not applicable.

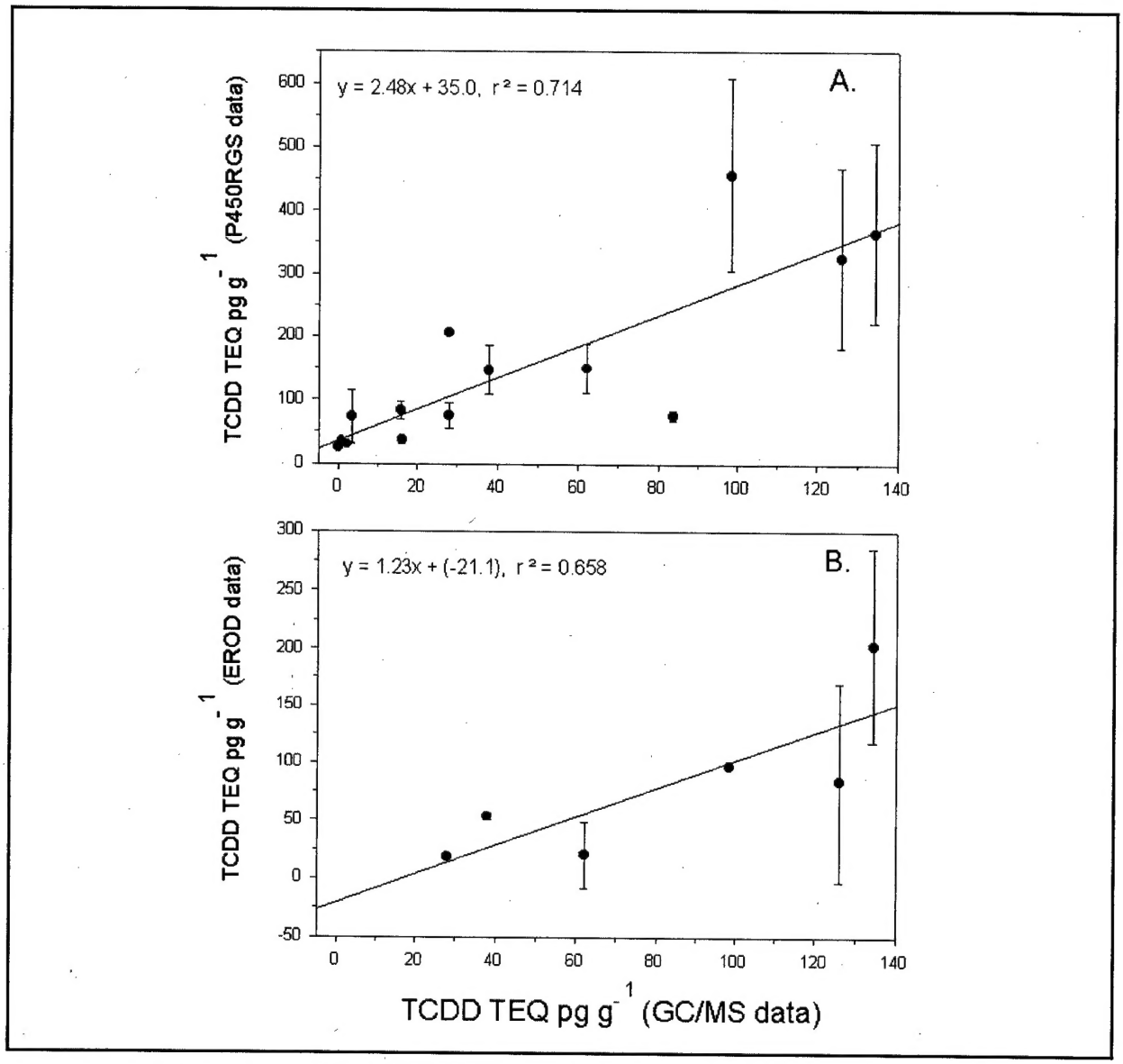

Figure 5. Correlation of calculated TCDD TEQs for GC/MS data with measured TCDD TEQs using the P450RGS assay (A) or the EROD assay (B) on 15 SA/SG-cleaned miscellaneous sediment extracts (Means of six replicates ± SD)

the GC/MS data was 0.658 (Figure 5B). Comparison of the six sediment extracts with detectable activity by both assays gave a correlation of 0.457 (Figure 6). As with the NYDMMP sediments, the P450RGS assay gave a higher response (about 2-fold) than did the EROD assay.

Interlaboratory Comparison of the P450RGS Assay. Splits of the NYDMMP sediment extracts were also analyzed by CAS in order to compare the reproducibility of results using the P450RGS assay. CAS performed the assay using only three replicates of each sample, whereas six replicates were analyzed at the U.S. Army Research and Development Center, Waterways Experiment Station (WES). The interlaboratory comparisons are shown in Figures 7 and 8 (crude and cleaned extracts, respectively). The correlation coefficients show that the results obtained by the two laboratories were very similar.

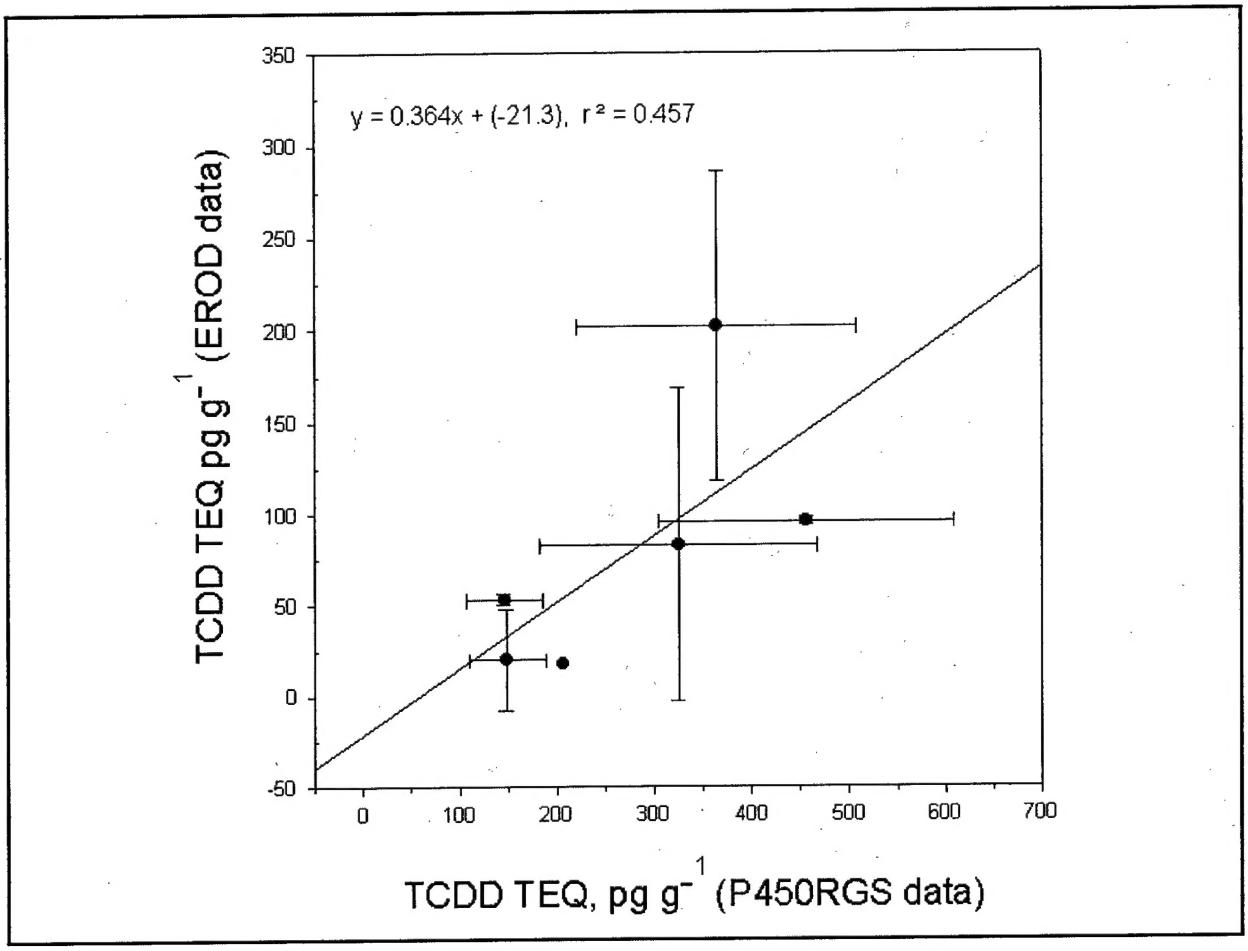

Figure 6. Correlation of P450RGS and EROD assays on 15 crude miscellaneous sediment extracts (Means of six replicates ± SD)

Time and Cost Comparisons. The P450RGS assay was less expensive than the EROD assay for testing the same number of samples. The cost of testing is reduced considerably when more samples are run at the same time. The P450RGS has significant advantages over the EROD assay in terms of the ease of performance and the time required for completion of the assay. Protein determination is not required for the P450RGS assay, and a much simpler instrument is used in the quantitation (i.e., a luminometer rather than a spectrofluorometer). The stronger signal produced by bioluminescence as compared with fluorescence increases the sensitivity of the P450RGS assay. The breakdown of cost and time requirements for the assays is shown in Table 2 below.

Table 2
Time and Materials Comparison of the P450RGS and EROD Assays

Expense Item*	P450RGS	EROD
Cost of supplies, $**	52	65
Labor time, hr↕	4.5	11.0
Protein determination	No	Yes

* Time and the cost of materials based on testing two samples of six replicates each.
** Direct costs only.
↕ To the nearest one-fourth hour.

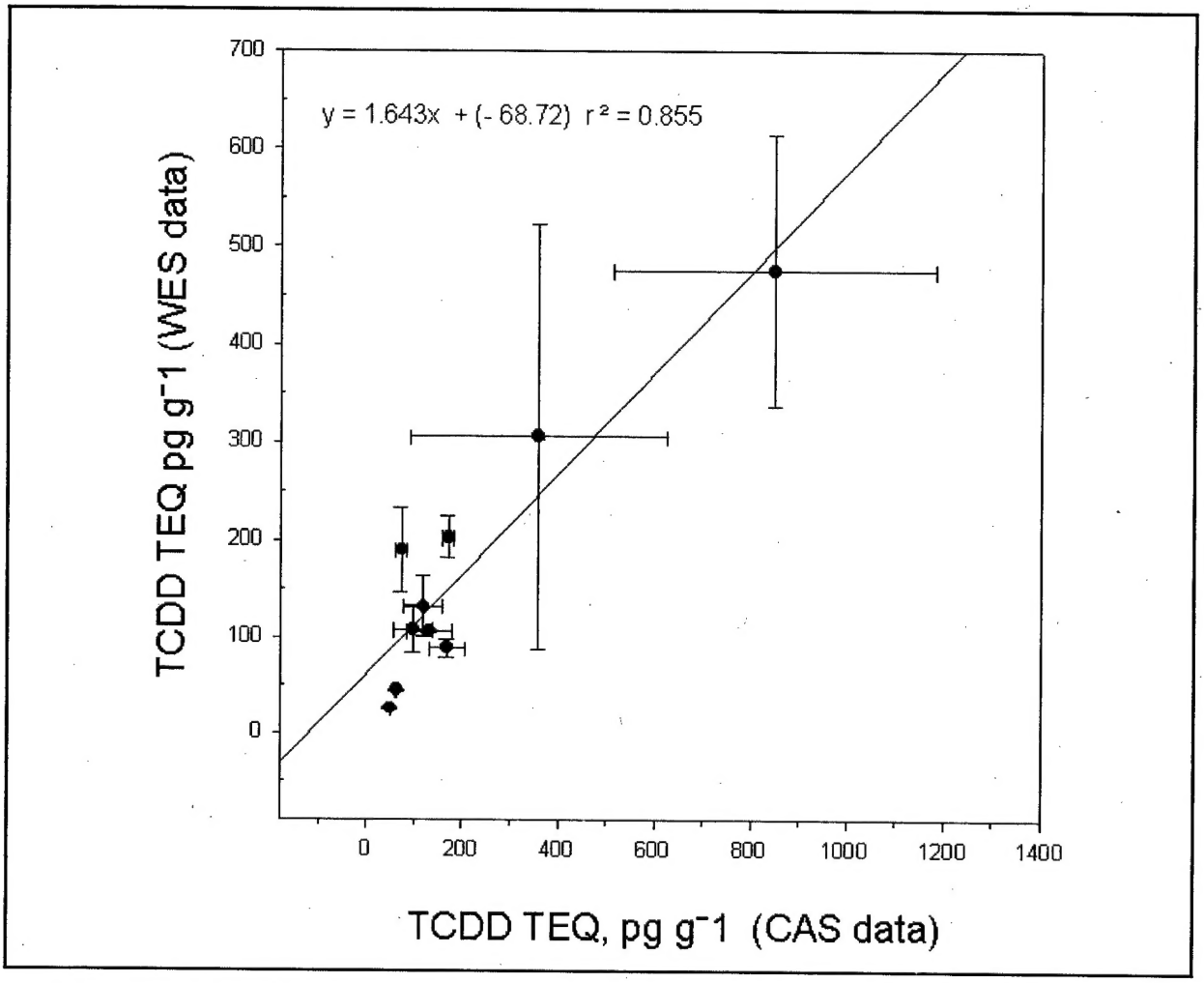

Figure 7. Correlation of P450RGS performed by WES and by CAS on 10 cleaned NYDMMP sediment extracts (WES data are means of six replicates ± SD; CAS data are means of three replicates ± SD)

CONCLUSIONS: The P450RGS and EROD assays are nearly equal in detecting dioxin and dioxin-like compounds in the sediments, but the P450RGS is the more sensitive of the two assays. In some cases in which the EROD assay could not detect the presence of dioxin and dioxin-like compounds in the sample, the P450RGS assay was able to do so. The P450RGS assay also has the capability of determining the predominance of either PAHs or dioxin/dioxin-like compounds without using a cleanup step, although more definitive results are obtained by using cleanup. The nonlabor costs incurred in testing two sediment samples are similar for the two assays, but the P450RGS is considerably less time-consuming.

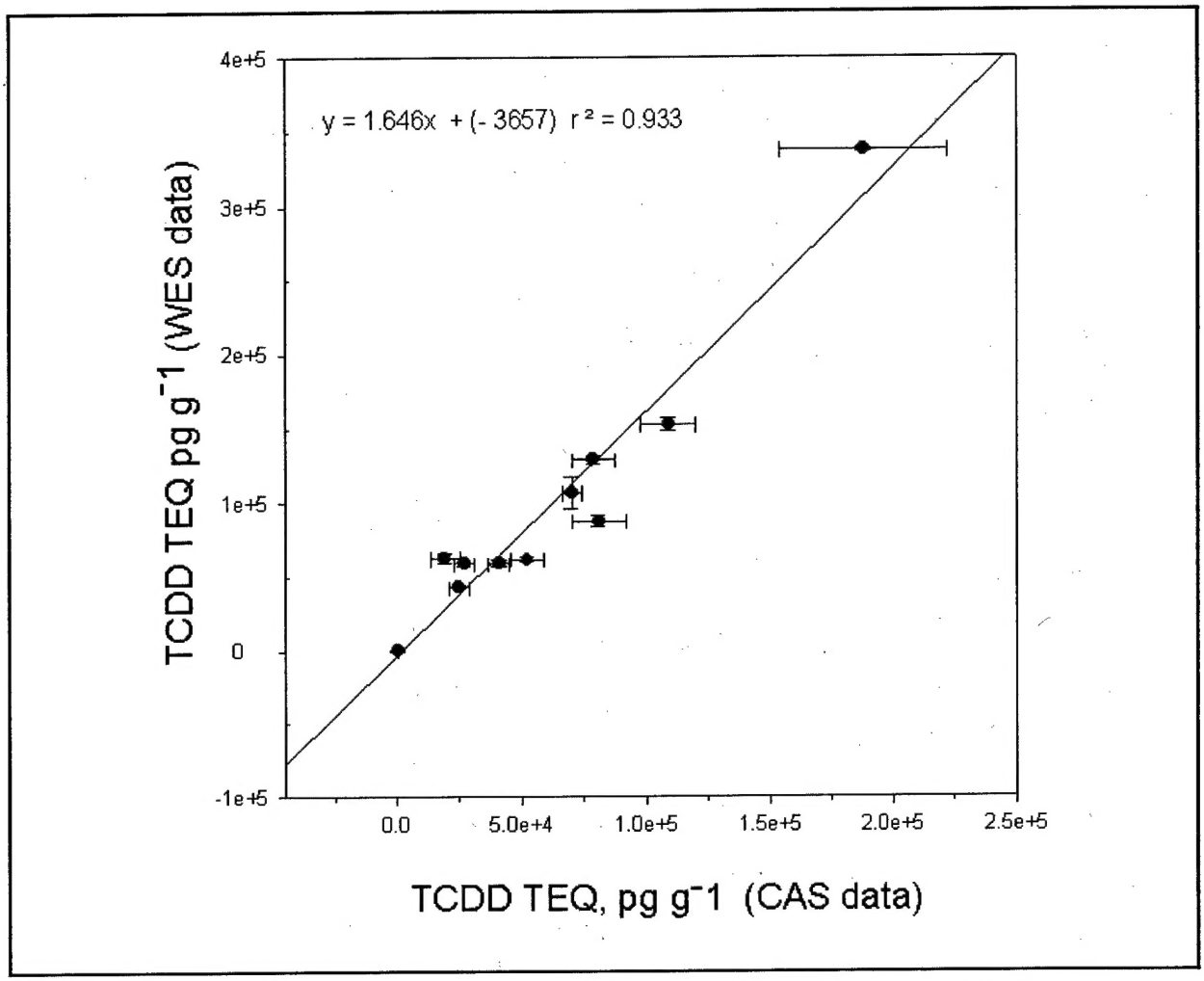

Figure 8. Correlation of P450RGS performed by WES and by CAS on 10 crude NYDMMP sediment extracts (WES data are means of six replicates ± SD; CAS data are means of three replicates ± SD)

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McFarland, V. A., Ang, C. Y., McCant, D. D., Inouye, L.S., and Jarvis, A. S. (1999). "Comparison of two cell-based assays for screening dioxin and dioxin-like compounds in sediments," *DOER Technical Notes Collection* (TN DOER-C8), U.S. Army Engineer Research and Development Center, Vicksburg, MS. *www.wes.army.mil/el/dots/doer*

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